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Cloning and characterisation of the *S.pombe rad15* gene, a homologue to the *S.cerevisiae RAD3* and human *ERCC2* genes

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ABSTRACT

The *RAD3* gene of *Saccharomyces cerevisiae* encodes an ATP-dependent 5′–3′ DNA helicase, which is involved in excision repair of ultraviolet radiation damage. By hybridisation of a *Schizosaccharomyces pombe* genomic library with a *RAD3* gene probe we have isolated the *S.pombe* homologue of *RAD3*. We have also cloned the *rad15* gene of *S.pombe* by complementation of radiation-sensitive phenotype of the *rad15* mutant. Comparison of the restriction map and DNA sequence, shows that the *S.pombe rad15* gene is identical to the gene homologous to *S.cerevisiae RAD3*, identified by hybridisation. The *S.pombe rad15.P* mutant is highly sensitive to UV radiation, but only slightly sensitive to ionising radiation, as expected for a mutant defective in excision repair. DNA sequence analysis of the *rad15* gene indicates an open reading frame of 772 amino acids, and this is consistent with a transcript size of 2.6kb as detected by Northern analysis. The predicted *rad15* protein has 65% identity to *RAD3* and 55% identity to the human homologue *ERCC2*. This homology is particularly striking in the regions identified as being conserved in a group of DNA helicases. Gene deletion experiments indicate that, like the *S.cerevisiae RAD3* gene, the *S.pombe rad15* gene is essential for viability, suggesting that the protein product has a role in cell proliferation and not solely in DNA repair.

INTRODUCTION

The ability to repair damaged DNA is an important requirement for cells to maintain chromosome integrity, and this is borne out by the existence of a number of human genetic disorders which result from defects in DNA repair. Cloning of human DNA repair genes has been achieved either directly by complementation of repair-deficient hamster or human mutants (1–7) or by virtue of their homology to DNA repair genes of lower eukaryotes (8,9). It has become apparent that fundamental molecular processes are highly conserved in eukaryotes, so that lower eukaryotes such as the yeasts provide powerful and easily manipulable model systems. The fission yeast, *Schizosaccharomyces pombe*, has recently become the focus of attention for its use as a simple model for eukaryotic organisms, e.g. in studies on the mechanism of cell cycle control (10).

In order to analyse the mechanisms by which DNA repair occurs, a large number of putative DNA repair mutants have been isolated from different organisms by virtue of their sensitivity to DNA damaging agents. In *Saccharomyces cerevisiae* at least 30 complementation groups have been identified and subsequently classified into three groups labelled the *RAD3*, *RAD52* and *RAD6* epistasis groups (reviewed in 11), which are either sensitive to UV radiation, gamma-irradiation or both UV and gamma-irradiation respectively. The *RAD3* group comprises genes required in an excision-repair pathway, and has been studied extensively, while the other two groups comprise genes required for recombination repair (*RAD52*) and error-prone (*RAD6*) pathways. DNA repair mutants have also been isolated in the distantly related yeast *S.pombe*, and assigned to 23 complementation groups (12,13). Existing data are, however, insufficient to permit an assignment of the mutants into different epistasis groups, but they have been classified according to their UV and/or gamma-radiation sensitive phenotypes (14). On this basis, the group 1 mutants display a UV sensitivity, which, as in wild type cells can be potentiated by caffeine (12) and the mutants may be described tentatively as being defective in an excision-repair pathway.

Complementation of the radiation-sensitive phenotypes has been used to isolate DNA repair genes from both species of yeast and

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from mammalian cells, and this has led to the identification of members of an excision-repair pathway which are conserved between *S.cerevisiae* and man. The *S.cerevisiae* genes *RAD3* and *RAD10* are homologues of the human *ERCC2* and *ERCC1* genes respectively (15,16). Reports to date on the cloning of DNA repair genes from *S.pombe*, as yet limited to members of the group 2 genes (*rad1*, *rad4* and *rad9* genes (17–19)), have not revealed homologies to any previously identified DNA repair genes. It was therefore of interest to determine whether *S.pombe* does contain pathways conserved in other eukaryotes (in this case excision repair) or whether the processes are different in this yeast.

The *S.cerevisiae RAD3* gene is required at an early stage in the excision-repair of ultraviolet-damage. Analysis of the *rad3* mutant has indicated that the gene product is required for nicking of DNA containing pyrimidine dimers (20). The gene encodes a single-stranded DNA-dependent nucleoside triphosphatase with DNA helicase (21) and DNA/RNA helicase activities (22, 23). As well as its role in excision-repair, the *RAD3* protein has an essential function for cell proliferation (24), and mutational analysis has revealed that different regions of the protein are involved in the repair and essential functions (25). The human *ERCC2* gene was cloned by its ability to correct the UV-sensitivity of hamster mutants in complementation group 2 (2). Sequencing of the *ERCC2* cDNA revealed that it was homologous to the *S.cerevisiae RAD3* gene (15).

In this study we report the cloning of an *S.pombe* gene showing 65% identity to the *S.cerevisiae RAD3* gene and 55% identity to the human *ERCC2* gene. This work provides evidence that at least one component of a nucleotide excision-repair pathway is conserved in *S.pombe*.

MATERIALS AND METHODS

Plasmids, strains and growth conditions

Plasmids used in this study: pUR19 (26) and the *S.cerevisiae RAD3*-containing plasmid, pNF3005 (a generous gift from Drs. W. Siede and E. Friedberg; 27). Two *S.pombe* genomic libraries were used; one contained DNA fragments partially digested with *Hin*dIII cloned into pDB262 (28), and the other contained DNA partially digested with *Sau*IIIA, cloned into pUR19 (26). *S.pombe* strains used were sp.074 (*rad15.P*, *leu1.32*, h^+) and sp.547 (*rad15.P*, *ade6.704*, *leu1.32*, *ura4.D18*, h^+) derived from *rad15.P* obtained from the Canadian National Repository; sp.008 (*ura1.61*, h^-), sp.011 (*leu1.32*, *ade6.704*, *ura4.D18*, h^-), 972 (h^-) from Prof. P.Nurse, Oxford, sp.101 (*leu1.32/leu1.32*, *ade6.704/ade6.704*, *ura4.D18/ura4.D18*, h^+/h^+) (19) and sp122 (*leu1.32/leu1.32*, *ade6.704/ade6.704*, *ura4.D18/ura4.D18*, *rad15/rad15::ura4* h^+/h^+), created in this study. *E.coli* strains were DH5α, (*F*$^-$, *endA1*, *hsdR17*, (*rk*$^-$, *mk*$^-$), *supE44*, *thi-1*, *recA1*, *lacIqZ-M15(lacproAB)*) and DH5αF′ (as DH5α but containing an integrated F′). Media for routine maintenance, transformation and sporulation of *S.pombe* were prepared as described by Gutz *et al.* (29) and Beach *et al.* (30).

Library screening

Library screening was carried out using ^{32}P-labelled random primed probes (31) for hybridisation under conditions of low stringency (55°C in 2×SSC for 16 h) and filters were exposed to Fuji-RX film.

General molecular biology methods

Plasmid and chromosomal DNA from *S.pombe* was purified according to the method of Aves *et al.* (32). DNA fragments were purified by extraction from low gelling temperature agarose using 'Geneclean' (Stratech Scientific Ltd.) according to the manufacturer's recommended methods. The nucleotide sequence was determined using Sequenase (US Biochemical Corp.) on DNA fragments subcloned into M13mp19. The sequence of both strands was determined using nested series of deletions created with exonuclease III (33). RNA was prepared from *S.pombe* using the method of Durkacz *et al.* (34), and poly A$^+$ RNA was purified using oligo-dT cellulose (35). Poly A$^+$ RNA was separated by electrophoresis on glyoxal phosphate agarose gels, transferred to Genescreen Plus (New England Nuclear Research Products) and probed by the manufacturer's recommended method. *E. coli* transformation was carried out as described by Hanahan (36). Other molecular biology techniques were as described by Sambrook *et al.* (35).

Yeast transformation and measurement of survival of yeast strains after UV or gamma irradiation

S.pombe transformation was performed according to the spheroplast method of Beach *et al.* (30). UV and gamma irradiation were carried out as described previously (19).

RESULTS

Identification of a homologue to the *S.cerevisiae RAD3* and human *ERCC2* genes

In order to determine whether *S.pombe* contains a homologue to the conserved *S.cerevisiae RAD3* and human *ERCC2* genes, an *S.pombe* genomic library in pDB262 was screened at low stringency with a *Sal*I fragment from pNF3005 (27). This fragment, which contains the *S.cerevisiae RAD3* gene minus the region encoding the acidic C terminus, was chosen to prevent cross-hydridisation with unrelated sequences encoding acidic

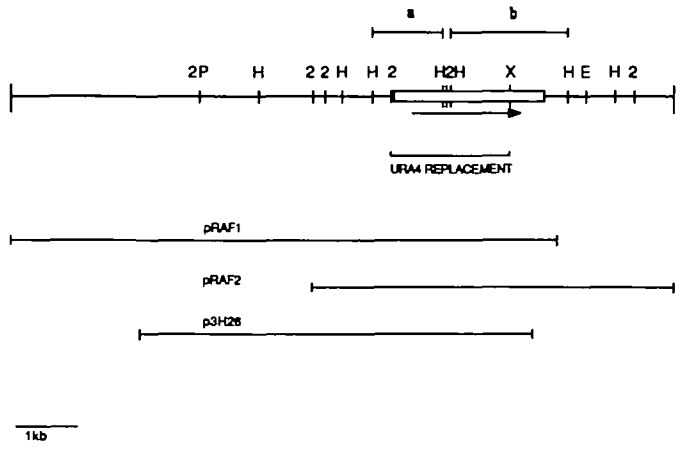

Figure 1. Restriction map of the *rad15* region. The open box indicates the position of the longest open reading frame, with the arrow indicating the direction of translation. E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; X, *Xba*I; 2, *Hin*dII. The extent of overlap between the plasmids: pRAF1, pRAF2 and p3H26 is depicted as line diagrams below the restriction map. The two fragments a and b, isolated by sequence homology, are show above the restriction map. The region replaced by the *ura4* gene in the gene disruption is also indicated.

amino acids. Five positive clones were studied in detail; four of them contained a *Hin*dIII fragment of 1.9kb and one had a 1.2kb *Hin*dIII fragment. These two sequences were used to identify a larger clone from another genomic library in the vector pUR19. The restriction map of this longer clone, p3H26, is shown in figure 1.

Isolation of the *S.pombe rad15* gene

In parallel to our search by hybridisation for homologues to *S.cerevisiae RAD* genes in *S.pombe*, we have been isolating *S.pombe* rad genes by complementation of *S.pombe* rad mutants (14, 18, 19). A genomic library in the vector pUR19 was used to transform the *rad15.P* mutant strain sp.547. Approximately 15,000 ura$^+$ transformant colonies were pooled, plated at a density of 10^5 cells per plate on selective medium and subjected to a UV dose of 100 Jm^{-2}. After two further rounds of selection, individual colonies were picked and checked for co-instability of the ura$^+$ and rad$^+$ phenotypes. DNA was then isolated from the UV resistant colonies and used to transform DH5 . Two plasmids were identified, pRAF1 and pRAF2 (fig 1). These were then retransformed into sp.547 and found to complement the *rad15.P* phenotype. Comparison of the restriction maps of the plasmids obtained by hybridisation and complementation indicated that there were restriction fragments common to all plasmids, suggesting that the same gene had been cloned by the two different methods.

Mapping of the gene to the *rad15* chromosmal locus

To confirm that the cloned gene was the *rad15* gene, its chromosomal location was mapped. pRAF2 was used to transform strain sp.547. A stable integrant (ura$^+$) was crossed to the strain sp.547. After sporulation no UV sensitive colonies that were ura$^+$, were seen confirming that the plasmid had integrated at the *rad15* locus.

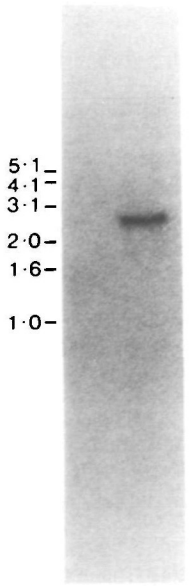

Figure 2. Northern analysis of the *rad15* transcript. 5 g polyA$^+$ RNA was probed with the 1.2 kb *Hin*dIII fragment from pRAF1. *Eco*RI *Hin*dIII digested single stranded lambda DNA was used as size markers (not shown). From this the estimated size of the transcript is 2.6kb.

The chromosomal location was further confirmed by pulse-field gel electrophoresis of *Not*1 digested *S.pombe* genomic DNA (37) and by genetic mapping. The *rad15.P* containing strain, sp.074, was crossed to the *ura1.61* containing strain sp.008. The segregation of the rad locus with respect to *ura1* was scored by random spore analysis. 154 non-segregants (73 rad$^-$ ura$^+$ and 81 rad$^+$ ura$^-$) and 10 segregants (7 rad$^+$ ura$^+$ and 3 rad$^-$ ura$^-$) were found. Thus the calculated distance between *rad15* and *ura1* is 6 +/− 3cM which is consistent with the published data (38).

Figure 3. Comparison of the protein sequences of the *S.pombe rad15*, *S.cerevisiae RAD3* and human *ERCC2* genes. RAD3, *S.cerevisiae RAD3*; ERCC2, human *ERCC2*; RAD15, *S.pombe rad15*. A dot indicates identity between *ERCC2* and the *RAD3* or *rad15* genes. Gaps have been introduced to maximise the alignment. The conserved helicase domains are underlined.

DNA sequence and Northern analysis

Sequence analysis of the insert DNA from plasmids obtained by hybridisation and complementation confirmed that the two methods had identified the same gene. Overall the sequence was determined for 3.16kb, comprising three *Hin*dIII fragments of 1.2, 0.06 and 1.9kb (fig 1). This sequence has been submitted to the EMBL database and has the accession number X60499.

The longest open reading frame encodes a protein of 772 amino acids with predicted M_r of 88,173 Da. No introns were identified either by analysis of the sequence for consensus splice sites or by comparison to the *S.cerevisiae RAD3* sequence. Northern analysis detects a transcript of 2.6kb (figure 2) which is consistent with the *rad15* open reading frame of 772 amino acids.

Comparison of the deduced amino acid sequence with that of the *S.cerevisiae RAD3* and human *ERCC2* proteins is shown in figure 3. The putative *rad15* protein has 65% identity to the *RAD3* protein and 55% identity to the *ERCC2* protein, while the *RAD3* and *ERCC2* proteins share 52% identity. The *S.cerevisiae RAD3* gene encodes an ATP-dependent DNA helicase (21), and by comparison with other helicases it has been shown to have the seven conserved helicase domains described by Gorbalenya *et al.* (39). The putative *rad15* protein also has these seven conserved domains, and in these regions the extent of the identity between the three proteins is particularly striking (60–85%), implying that the putative *rad15* protein is also a DNA helicase. One of these domains contains the consensus sequence for an NTP-binding motif (GXGKT), at amino acids 45–49. This motif, and the regions immediately adjacent to it are highly conserved between the three proteins. This is of interest as Sung *et al.* (21) have used mutagenesis of the conserved lysine residue to show that this region is essential for the DNA-repair function of the *RAD3* protein. The *RAD3* protein has an acidic C terminal which is not present in the *ERCC2* protein, in this respect the C terminal of the *rad15* protein is intermediate between *RAD3* and *ERCC2*.

Gene deletion

A null allele was constructed to determine whether the *rad15* gene was essential for viability. A construct was made in which a *Hin*dII-*Xba*I fragment, containing the majority of the ORF including the ATG, was replaced by the *ura4* gene (40) using the partial fill-in method described by Barbet *et al.*, (26). A linear fragment spanning the gene from an upstream *Hin*dII site to a downstream *Eco*RI site (see Fig.1) was then used to transform the diploid strain sp.101 to uracil prototrophy. Integration was checked by Southern analysis and an h^{90} (sp.122) derivative sporulated on low nitrogen plates. No ura^+ colonies were recovered by random spore analysis and tetrad analysis showed only 2 of the 4 spores per ascus were viable. These spores were all ura^-, indicating that the *rad15* gene is essential for viability.

Complementation data and radiation sensitivities of the *rad15* mutant

The identification of the *S.pombe* homologue to the *S.cerevisiae RAD3* and human *ERCC2* genes as the *S.pombe rad15* gene allows an analysis of the phenotype of cells mutated in this gene. The *rad15* mutant is very sensitive to UV radiation (0.0025% survival after exposure to 250 J/m^2) as compared to wild type (20% survival after the same dose) as shown in figure 4, but less sensitive to ionising radiation (18% for *rad15* compared to 50% for the wild type after exposure to 1000 Gy). The *rad15* phenotype is similar to those of the majority of the mutant alleles of the *S.cerevisiae RAD3* gene (24,27).

The full length clones pRAF1 and pRAF2 complement both the UV and gamma-irradiation sensitivities to virtually wild type

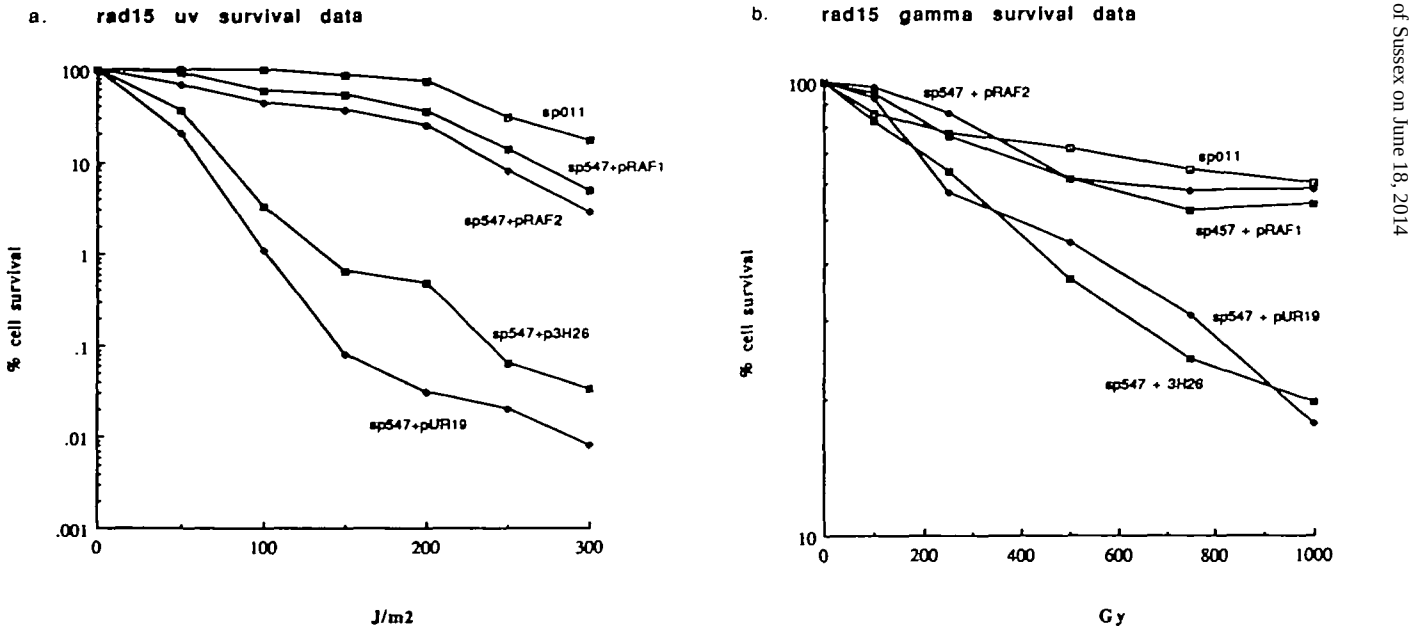

Figure 4. Survival curves of *S.pombe* strains following UV and Gamma irradiation. a. Response to UV radiation of the *S.pombe* strains sp.011 and sp.547, and the pUR19, pRAF1, pRAF2 and p3H26 transformants of sp.547. b. Response to gamma irradiation of the same strains and transformants. Note the difference in scale of the UV-survival and the gamma-survival.

levels. In contrast, the truncated clone identified by hybridisation, p3H26, which is missing approximately 50 amino acids at the C terminus, does not complement the *rad15* mutation.

DISCUSSION

Comparison of DNA repair mechanisms between *S.cerevisiae* and man shows that a number of genes required for a nucleotide excision-repair pathway are conserved between these two organisms (41), but to date, little information has been available as to whether a similar mechanism exists in *S.pombe*. We show here that the *S.pombe rad15* gene product has a high degree of identity to the *S.cerevisiae RAD3* and human *ERCC2* proteins, showing that this component of the excision repair pathway is highly conserved between these three evolutionarily diverged species. Moreover the *S.cerevisiae RAD3* gene complements the UV radiation sensitive phenotype of the *rad15.P* mutation, and an *S.pombe* homologue of the *RAD3* gene cloned independently by S.Prakash and co-workers complements the excision repair defect in a *RAD3* mutant (S.Prakash, pers. comm.) showing that the function of this gene is conserved.

In both *S.cerevisiae* and *S.pombe* the genes are essential for viability, indicating that the gene products have a role in cell proliferation and not solely in DNA repair. The high level of sequence homology suggests that the *S.pombe* gene is also likely to encode an ATP-dependent DNA helicase. Within a superfamily of DNA and RNA helicases, Gorbalenya *et al.* (39) have identified several distinct families as defined by the spacing and sequence of seven conserved domains. Within these families the *RAD3* protein is the least easy to categorise since both the homology of the domains and the spacing between them are different from that observed for other members of the superfamily. It is thus interesting to note that comparison of the *S.cerevisiae RAD3* protein sequence with those of the human *ERCC2* and *S.pombe rad15* proteins indicates a high degree of similarity not only within the domains but also of the spacing and sequence between the domains. This supports the view that the three genes are true homologues and not simply members of the same superfamily. The sequence conservation outside the known helicase domains may highlight regions important for the specific roles of the proteins.

A number of other helicases have been identified in *S.cerevisiae* as having roles in DNA repair, for example the *RADH* gene which is associated with a mutagenic DNA repair pathway (42–44). However, in comparison to the *RAD3* gene homologues these helicases appear to be members of a separate gene family comprising the *E. coli uvrD*, *rep*, *recB* and *recD* genes (39, 43). Additionally the human *ERCC3* and *ERCC6* gene products are also proposed to be helicases, due to the presence of the putative helicase functional domains (4, and C. Troelstra, cited in 45).

A number of different mutant alleles of the *S.cerevisiae RAD3* gene have been isolated. The *rad3-1* and *rad3-2* alleles are highly sensitive to UV radiation (24, 27) and biochemical studies have indicated that the alleles are defective in a component required for excision-repair (20, 46). In contrast the *rem1-1* and *rem1-2* mutants, which map within the *RAD3* gene are not defective in excision-repair, but confer a semi-dominant hyper-recombination/hyper-mutation phenotype and have been proposed to be defective in a mismatch-repair pathway or DNA replication (47). In comparison, the *S.pombe rad15.P* mutant is also highly sensitive to UV radiation but only slightly sensitive to gamma-irradiation, in much the same way as the *rad3-1* and *rad3-2* alleles of *S.cerevisiae*, indicating a role for *rad15* in an excision repair pathway.

Recent data of Weber *et al.* (cited in 45) have shown that the *ERCC2* gene is able to correct the UV-sensitivity of cells from patients with xeroderma pigmentosum complementation group D. Cells from these patients are deficient in the excision repair pathway (48).

Our knowledge of the extent of conservation of excision repair genes between *S.cerevisiae*, *S.pombe* and man has recently been extended by the identification of additional *S.pombe* homologues to *S.cerevisiae* genes. We have identified homologues to *S.cerevisiae rad1* and *rad2* (14). Further evidence for the conservation of excision repair pathways is provided by novel homologues to the human excision repair gene *ERCC3*, which have been identified in both yeasts (41, Koken *et al.* cited in 45). Other repair pathways have yet to be identified as conserved between the two yeasts or between either yeast and man. However, homologues of the *RAD6* gene of *S.cerevisiae*, involved in error-prone repair and coding for a ubiquitin-conjugating enzyme (49), have been isolated from *S.pombe* (50), *Drosophila* (51), and humans (8, 9). It is thus evident that many DNA repair genes are highly conserved in eukaryotes.

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